FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
[redacted]-PCT-US 99	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
60/122,312	09/914541

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/05158	01, March 2000	01, March 1999

TITLE OF INVENTION

EUKARYOTIC PEPTIDE UPTAKE SYSTEM FOR TRANSPORTATION OF ENKEPHALINS

APPLICANT(S) FOR DO/EO/US

BECKER, Jeffrey M., HAUSER, Melinda, DONHARDT, Amy, BARNES, David

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

POSTCARD, FIGURES 16A & 16B

Rec'd PCT/PTO 29 AUG 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/914541	PCT/US00/05158	1046-PCT-US-00

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☒	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$690.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	18 - 20 =	0	x $18.00	**$0.00**
Independent claims	5 - 3 =	2	x $80.00	**$160.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**
	TOTAL OF ABOVE CALCULATIONS =			**$850.00**
☒	Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.			**$425.00**
	SUBTOTAL =			**$425.00**
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +			**$0.00**
	TOTAL NATIONAL FEE =			**$425.00**
☐	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			**$0.00**
	TOTAL FEES ENCLOSED =			**$425.00**
			Amount to be: refunded	$
			charged	$

a. ☒ A check in the amount of **$425.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **13-3405** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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33,167
REGISTRATION NUMBER

August 29, 2001
DATE

EUKARYOTIC PEPTIDE UPTAKE SYSTEM FOR TRANSPORTATION OF ENKEPHALINS

RELATED CASES

This application is a conversion of provisional application Serial No. 60/122,312, filed on March 1, 1999, entitled: Enkephalins are Transported by a Novel Eucaryotic Peptide Uptake System.

FIELD OF THE INVENTION

The invention relates to plant molecular genetics, and more specifically, to an oligopeptide transporter in the yeast *Saccharomyces cerevisiae*. The transporter mediates the uptake of tetra- and penta- peptides, including leucine enkephalin and methionine enkephalins.

BACKGROUND OF THE INVENTION

Peptide uptake is the process by which individual cells are able to transport intact peptides across their plasma membranes. The process is a general physiological phenomenon of bacteria, fungi, plant cells and mammalian cells (Becker, J. M. et al, In: Microorganisms and Nitrogen Sources, Payne, J. W. (ed.), John Wiley and Sons, Inc., pp. 257-279 (1980); Matthews, D. M. et al., Curr. Top. Membr. Transp. 14:331-425 (1980)). In every case studied so far, peptide transport is a specific biochemical process in which small peptides ($\leq$ 6 amino acids) are transported across a membrane by energy-dependent, saturable carriers.

Three genetically distinct systems of peptide uptake have been identified in gram-negative bacteria. An oligopeptide permease (Opp) system has been identified in bacteria such as *E. coli*, and *S. typhimurium* (Andrews, J. C. et al., J. Bacteriol. 161:484-492 (1985); Hogarth, B. G. et al., J. Bacteriol. 153:1548-1551 (1983)). The Opp system is capable of transporting peptides having up to 5 amino acid residues, regardless of their side chains (Payne, J. W. et al, J. Biol. Chem. 243:3395-3403 (1968); Payne, J. W. et al., J. Biol. Chem. 243:6291-6299 (1968)). In contrast, tripeptide permease (Tpp) systems, such as that of *S. typhimurium*, exhibit an apparent affinity for peptides having hydrophobic amino acid residues (Gibson, M. M. et al., J. Bacteriol. 160:122-130 (1984)). The third system, a dipeptide permease (Dpp) system, has a preference for transporting dipeptides (Abouhamad, W. N., et al., Mol. Microbiol. 5:1035-1047 (1991)). Functionally similar systems have been described in fungi and yeast (Naider, F. et al., In: Current Topics in Medial Mycology, volume II, McGinnis, M. M. (ed.) (1987)), but have not been well characterized.

The genes that encode the protein components of the oligopeptide transporters of *E. coli* (Kashiwagi, K. et al, J. Biol. Chem. 265:8387-8391 (1990)), *Salmonella typhimurium* (Hiles, I. D. et al., Eur. J. Biochem. 158:561-567 (1986); Hiles, I. D. et al, J. Molec. Biol 195:125-142 (1987)), *Bacillus subtilis* (Rudner, D. Z. et al., J. Bacteriol. 173:1388-1398 (1991); Perego, M. et al., Mol. Microbiol. 5:173-185 (1991)), *Streptococcus pneumoniae* (Alloing, G. et al., Mol. Microbiol. 4:633-644 1990)), and *Lactococcus lactis* as well as two dipeptide permeases, one in *E. coli* (Abouhamad, W. N., et al., Mol. Microbiol. 5:1035-1047 (1991)), and the other in

Bacillus subtilis (Mathiopoulos, C. et al., Mol. Microbial. 5:1903-1913 (1991)) have been cloned and sequenced.

The ability of bacteria and plant cells to accumulate peptides has been found to be dependent upon peptide transport systems (Becker, J. M. et al., In: Microorganisms and Nitrogen Sources, Payne, J. W. (ed.), John Wiley and Sons, Inc., pp. 257-279 (1980); Matthews, D. M. et al., Curr. Top. Membr. Transp. 14:331-425 (1980); Higgins, C. F. et al., In: Microorganisms and Nitrogen Sources, Payne, J. W. (ed.), John Wiley and Sons, Inc., pp. 211-256 (1980); Naider, F. et al., In: Current Topics in Medial Mycology, volume II, McGinnis, M. M. (ed.) (1987)). These systems are distinct from the mechanisms that mediate the uptake of amino acids.

The existence of peptide transport systems in plants was demonstrated by showing that plants could accumulate non-hydrolyzable, non-physiological peptide substrates, intact and against a concentration gradient (Higgins, C. F. et al., Planta 134:205-206 (1977); Higgins, C. F. et al., Planta 136:71-76 (1977); Higgins, C. F. et al, Planta 138:211-216 (1978); Higgins, C. F. et al., Planta 142:299-305 (1978); Sopanen, T. et al., FEBS Lett. 79:4-7 (1977)). The transport system was found to exhibit saturation kinetics and to be inhibited by a range of metabolic inhibitors (Higgins, C. F. et al., Planta 136:71-76 (1977)). The plant peptide transport system can transport both di- and tripeptides (Sopanen, T. et al., FEBS Lett. 79:4-7 (1977); Higgins, C. F. et al., Planta 142:299-305 (1978)). Plant peptide transport systems are capable of transporting a wide variety of peptides. These systems exhibit broad transport specificity with respect to amino acid side-chains. The presence of D-amino acids, however, reduces the transport rate, thus indicating that the transporters have

strong stereospecificity. Two proteins, approximately 66 D and 41 D, have been suggested as components of the plant peptide transport system in barley grains (Payne, J. W. et al., Planta 170:263-271 (1987).

The primary function of peptide transport is to supply amino acids for nitrogen nutrition (Payne, J. W. et al., In: Microorganisms and Nitrogen Sources, Payne, J. W. (ed.), John Wiley and Sons, Inc., pp. 257-279 (1980); Matthews, D. M. et al., Curr. Top. Membr. Transp. 14:331-425 (1980); Becker, J. M. et al., In: Microorganisms and Nitrogen Sources, Payne, J. W. (ed.), John Wiley and Sons, Inc., pp. 257-279 (1980); Adibi, S. A. et al., Metabolism 36:1001-1011 (1987); Higgins, C. F. et al., Planta 138:211-216 (1978); Sopanen, T. et al., FEBS Lett. 79:4-7 (1977); Higgins, C. F. et al., Planta 138:217-221 (1978)). In bacteria, peptide transport has, however, also been associated with sporulation (Perego, M. et al., Mol. Microbiol. 5:173-185 (1991); Mathiopoulos, C. et al., Mol. Microbiol. 5:1903-1913 (1991)); chemotaxis (Manson, M. D. et al., Nature 321:253-256 (1986), and the recycling of cell wall peptides (Goodell, E. W. et al., J. Bacteriol 169:3861-3865 (1987)).

Small peptides containing four to five amino acid residues are transported by a recently identified class of peptide transporters named the OPT1 family (Lubkowitz et al. Mol. Microbiol. (1998) 28(4):729-741, incorporated herein by reference). The amino acid sequence of this family is distinct from that of the PTR family, a ubiquitous group of proton-coupled transporters which selectively transports di- and tripeptides. Phylogenetic analysis suggests that the OPT family is also distinct from the major facilitator superfamily (MFS), a diverse collection of proteins which

catalyzes the transport of a wide variety of substrates, including sugars, amino acids, neurotransmitters, and drugs.

Members of the OPT family have been identified and characterized in the yeasts *Candida albicans*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. Additional members exist in plants, as indicated by searches of publicly accessible data bases. In mammalian tissues, reports in the literature suggest that the enkephalins, endogenous pentapeptides involved in analgesia in the central nervous system, are transported across the blood-brain barrier by a specific, saturable transport system. The existence of enkephalin transporters has been inferred from data obtained by measuring whole brain flux of the peptides in rodents. To date, no protein has been identified in eukaryotes as the discrete enkephalin carrier.

SUMMARY OF THE INVENTION

It has now been discovered that the endogenous opioids Met-enkephalin and Leu-enkephalin, pentapeptides of amino acid sequence YGGFM and YGGFL, respectively, can be transported by cells expressing the *S. cerevisiae* ORF YJL212C. When expressed under the control of a constitutive promoter in a high copy number vector, this OPT family member is necessary and sufficient to transport Leu-enkephalin into yeast cells. This is the first example of a genetically defined eukaryotic transport protein which can transport enkephalins across the cell membrane. This gene has been named OPT1.

The invention also comprises a method for obtaining mammalian enkephalin transporters by functional complementation of OPT1 deficient yeast.

The invention also comprises a fungicidal composition comprising a toxic analogue of enkephalin as an active ingredient, and a method of killing fungi comprising applying a toxic analogue of enkephalin to a substrate or organism to be treated.

The invention also comprises a method of preventing or reducing fungal growth on substrates.

The invention also comprises OPT1 transformed plants, methods of such transformation and methods of growing transformed plants.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows shows the growth of *S. cerevisiae* BY4730 expressing OPT family members.

Fig. 2 shows uptake of [^{3}H]Leu-enkephalin by *S. cerevisiae* BY4730 transformants.

Fig. 3 shows a chromatographic analysis of Leu-enkephalin.

Fig. 4 shows the effects of naloxone and naltrexone on the uptake of [^{3}H]Leu-enkephalin and [^{3}H]leucyl-leucine.

Fig. 5 shows the amino acid sequence of the OPT family member (isp4-like protein) from *Arabidopsis thaliana* designated emb CAB43855.1

Fig. 6 shows the amino acid sequence of the OPT family member (isp4-like protein) from *Arabidopsis thaliana* designated emb CAB10414.1

Fig. 7 shows the amino acid sequence of the OPT family member(isp4 like protein) from *Arabidopsis thaliana* designated Genbank GB:D14061.

Fig. 8 shows the amino acid sequence of the OPT family member (previously unknown protein) from *Arabidopsis thaliana* designated emb CAB38285.

Fig. 9 shows the amino acid sequence of the OPT family member (isp4 like protein) from *Arabidopsis thaliana* designated Genbank GB:D83992.

Fig. 10 shows the amino acid sequence of the OPT family member (Opt1p) from *Candida albicans* designated Genbank GB:AAB69628.1.

Fig. 11 shows the amino acid sequence of the OPT family member (Opt1) from *Saccharomyces cerevisiae* designated YJL212c.

Fig. 12 shows the amino acid sequence of the OPT family member (YPR194c) from *Saccharomyces cerevisiae* designated YPR194c.

Fig. 13 shows the amino acid sequence of the OPT family member (previously unknown protein) from *Schizosaccharomyces pombe* designated emb CAB16254.1.

Fig. 14 shows the amino acid sequence of the OPT family member from *Schizosaccharomyces pombe* designated emb CAA19062.1.

Fig. 15 shows the amino acid sequence of the OPT family member from *Schizosaccharomyces pombe* designated sp40900 or Gi 729859.

Fig. 16 shows a sequence comparison of Opt1 family members.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention arises, in part, from the exploitation of the *S. cerevisiae* OPT1p. *S. cerevisiae* strains that carry mutations in OPT1 may be created by knocking out *OPT1* by any means known in the art, such as homologous recombination with a defective *OPT1*, by homologous recombination replacing *OPT1* with another gene, by mutation and selection, and the like. These knock-out yeasts will be completely deficient for enkephalin transport, which can be determined by

their resistance to toxic derivatives of enkephalin and lack of uptake of radiolabeled Met- and Leu-enkephalin.

In addition to obtaining mammalian homologues to *OPT1* by functional complementation, functional augmentation may also be used. In all the wild-type yeast strains tested, enkephalin transport was deficient in the absence of a strong promoter such as the ADH promoter. Therefore, yeast vectors carrying exogenous mammalian DNA may be transformed into wild-type yeast and examined for enkephalin transport. Preferably, these vectors comprise a strong promoter to augment expression of a functional enkephalin transport protein.

Thus, one aspect of the present invention is the construction of a stable *S. cerevisiae OPT1* mutant. Methods for isolating such mutants are described below, and by Perry, J. R. et al. (In: "Isolation and Characterization of a *Saccharomyces cerevisiae* Peptide Transport Gene," Molecular and Cellular Biology, volume 14 (1994), herein incorporated by reference in its entirety). Polynucleotides that encode the peptide transport genes of higher plants have been identified and isolated by their capacity to complement the peptide transport deficiency of the stable *S. cerevisiae* ptr2 strain. In a like manner, knock-out S. cerevisiae may be used in functional complementation assays using polynucleotides that encode mammalian proteins. Transformation of knock-out *S. cerevisiae* with mammalian sequences and subsequent growth on Leu-enkephalin as the source of leucine may reveal mammalian homologues of *OPT1*. In addition, functional augmentation may be employed to obtain mammalian homologues.

The present invention relates in part to the isolation of a novel polynucleotide that is capable of hybridizing to, or recombining with, a plant gene that encodes a peptide transport protein. The polynucleotides of the present invention are "substantially purified," in that they have been purified from undesired yeast genes with which they are associated in nature. The molecules maybe in either a double-stranded or single-stranded form. Such polynucleotides are capable of augmenting the transport capacity of a recipient plant, and thus may be used to facilitate the delivery of desired compounds to the plant. In an alternative embodiment, the polynucleotides of the present invention can be used to disrupt or otherwise inactivate endogenous transport systems. Such disruption renders the plant incapable of transporting toxic peptides, and thus resistant to pathogens that produce such peptides.

The capacity of the polynucleotides of the present invention to hybridize to a plant gene arises out of the extent of homology between the respective sequences of the polynucleotides. As used herein, a polynucleotide of the present invention is said to be able to "hybridize" to a plant gene if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. The molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Such conventional stringency conditions are described by Sambrook, J., et al., (In:

Molecular Cloning, a Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)), and by Haymes, B. D., et al. (In: Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C. (1985)), both herein incorporated by reference).

Complementary molecules thus need not exhibit "complete complementarity," but need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure. Departures from complete complementarity are, therefore, permissible, so long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In contrast, where two nucleic acid molecules exhibit "complete complementarity," every nucleotide of one of the molecules is complementary to a nucleotide of the other; such molecules need not have the same lengths.

The capacity of the polynucleotides of the present invention to recombine with a plant gene is determined by the extent of sequence "homology" between the polynucleotide and the plant gene. Homologous recombination is a well-studied natural cellular process which involves the exchanges of a region of one polynucleotide with a region of another (see, Sedivy, J. M.,Bio-Technol. 6:1192-1196 (1988)). Sufficient homology for recombination requires only minimal homology in regions of the polynucleotide that flank the portion of the polynucleotide that undergoes recombination. The region may be of any length from a single base to a substantial fragment of a chromosome. Generally, a region having a length of about ten nucleotide residues is sufficient. Recombination is catalyzed by enzymes which are naturally present in both prokaryotic and eukaryotic cells.

The polynucleotides of the present invention comprise isolated nucleic acid molecules that can complement or augment a tetra or pentapeptide transport deficiency of *S. cerevisiae*. The term "polynucleotide" encompasses nucleic acid molecules that encode a complete protein, as well as nucleic acid molecules that encode fragments of a complete protein. The polynucleotides may comprise the wild-type allele (or a portion of such allele) of a functional peptide transport gene, or they may comprise mutated or disrupted (as by the insertion of additional DNA or RNA) alleles of such genes. As used, herein a "fragment" of a polynucleotide is an oligonucleotide whose nucleotide sequence is identical to that of a region of the polynucleotide, and whose length is greater than about 15 nucleotide residues, and preferably greater than about 20 nucleotide residues.

Functional complementation or augmentation

The isolation and cloning of polynucleotides that encode *S. cerevisiae* enkephalin transport proteins permits the isolation of analogous, complementary polynucleotides from mammalian cells. The functional role of such isolated polynucleotides can be readily determined by transforming them into the above-described stable enkephalin transport-deficient yeast strain, and evaluating whether transformants acquire the capacity to transport intact enkephalin. Thus, the methods of the present invention permit the isolation of polynucleotides from mammalian cells. Such polynucleotides are the equivalents of the preferred polynucleotides of the present invention.

In one embodiment of the invention, mammalian protein encoding sequences are cloned into suitable yeast expression vectors. Such vectors comprise regulatory

sequences such as promoters, termination signals and restriction endonuclease recognition sequences to permit the introduction of heterologous sequences, such as the mammalian protein encoding sequences. Any suitable vector for expression of proteins in yeast known in the art may be used.

Examples of suitable yeast vectors include the yeast 2-micron circle, the expression plasmids YEP13, YCP and YRP, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al., Miami Wntr. Syrup. 19:265-274 (1982); Broach, J. R., In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 445-470 (1981); Broach, J. R., Cell 28:203-204 (1982); Sherman, F. et al., In: Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1986)).

Yeast transformation may be accomplished by any means known in the art. Selection of transformed yeast with functional complementation or augmentation of the deleted OPT1 may be on enkephalin containing media as the source of leucine, for example.

Yeast transformants that display functional complementation or augmentation may be further analyzed by isolating the heterologous DNA and determining the nucleic acid sequence. The deduced amino acid sequence may be analyzed for identity and/or similarity by any of the available sequence analysis programs. A score of high identity or similarity, and predicted structural features of the deduced amino acid sequence (particularly the presence and number of transmembrane domains and the presence of the putative consensus sequence for OPT1 family members) indicates homology of the mammalian sequence to *S. cerevisiae* OPT1p.

For complementation, OPT1 knock-out yeast strains may be generated and used. Alternatively, wild-type yeast may be used in augmentation studies. In either case, yeast cells are transformed with yeast expression vectors carrying heterologous mammalian DNA. Preferably, in augmentation studies, the expression of the heterologous sequence in the yeast expression vector is under the control of a strong promoter, such as the ADH promoter.

Antifungal Compositions

Another embodiment of the inention comprises an antifungal composition comprising at least one toxic derivative of enkephalin as an active ingredient. Another embodiment is a method of using the antifungal compositions comprising applying the antifungal composition to a substrate to reduce or prevent fungal growth.

There is a plethora of toxic moieties that may be employed in the antifungal composition and method using the antifungal composition. Virtually any toxic moiety may be used that satisfies the following criteria: the toxic moiety or moieties must be able to be associated with the enkephalin peptide, and the moiety or moieties must not interfere with the uptake of the peptide into the cells.

Substantial evidence suggests that the uptake of toxic peptides is mediated by peptide transport systems (McCarthy, P. J. et al.,Antimicrob. Agents Chemother. 28:494-499 (1985); McCarthy, P. J. et al., J. Gen. Micro. 131:775-780 (1985); Moneton, P. et al., J. Gen. Micro. 132:2147-2153 (1986); Yadan, J. C. et al., J. Bacteriol. 160:884-888 (1984)); Payne, J. W. et al., FEMS Microbiol. Letts. 28:55-60 (1985); Mehta, R. J. et al., Antimicrob. Agents Chemother. 25:373-374 (1984)). Since the polynucleotides of the present invention define the genetic loci responsible

for enkephalin transport in yeast, fungi would be take up toxic enkephalin derivatives by transport, and fungal growth would be prevented or reduced.

In this regard, the present invention provides a method for conjugating an antimicrobial or antifungal agent or a pesticide to a peptide in order to provide a more effective treatment against fungal growth. In a similar manner, toxic peptide derivatives maybe used as herbicides to eliminate fungal growth around plants (particularly crops).

Examples of toxic peptide or peptidyl molecules that may be used include, but are not limited to:

(A) metabolic toxins (such as the antifungal agent FMDP ›N.sup.3 -(4-methoxyfumaroyl)-L-2,3 diaminopropanoic acid), toxic nucleotides (such as halogenated nucleotides (e.g., 5-fluoroorotic acid), dideoxynucleotides, mutagenic nucleotide or nucleoside analogs, etc. (Kingsbury, W. D. et al., J. Med. Chem. 27:1447-1451 (1984); Andruszkiewicz, R. et al., J. Med. Chem.30:1715-1719 (1987); Andruszkiewicz, R. et al., J. Med. Chem. 33:132-135 (1990); Andruszkiewicz, R. et al., J. Med.Chem. 33:2755-2759 (1990); Milewski, S. et al., J. Drugs Expt. Clin. Res. 14:461-465 (1988));

(B) peptides that contain toxic amino acids (such as oxalysine, fluorophenylalanine, ethionine, unusual D amino acids, etc.)(McCarthy, P. J. et al., Antimicrob. Agents Chemother. 28:494-499 (1985); Marder, R. et al., J. Bacteriol. 36:1174-1177(1978); Moneton, P. et al., J. Gen. Micro. 132:2147-2153 (1986); Mehta, R. J. et al., Antimicrob. Agents Chemother.25:373-374 (1984); Bosrai, M. et al., J. Gen. Microbiol. 138:2353-2362 (1992));

(C) toxic peptides and peptidyl molecules such as bacilysin (Milewski, S. et al., Arch. Microbiol. 135:130-136 (1983);Moneton, P. et al., J. Gen. Microbiol. 132:2147-2153 (1986); Kenig, M. et al. J. Gen. Microbiol. 94:37-45 (1976)), polyoxins (especially polyoxin D) (Becker, J. M. et al., Antimicrob. Agents Chemother. 23:926-929 (1983)), nikkomycins(especially nikkomycin Z) (Dahn, U. et al., Arch. Microbiol. 107:143-160 (1976)), and their analogs (Smith, H. A. et al.,Antimicrob. Agents Chemother. 29:33-39 (1986); Naider, F. et al., Antimicrob. Agents Chemother. 24:787-796 (1983);Krainer, E. et al., J. Med. Chem. 34:174-180 (1991); Shenbagamurthi. P. et al., J. Med. Chem. 26:1518-1522 (1983); Shenbagamurthi. P. et al., J. Med. Chem. 29:802-809 (1986); Khare, R. K. et al., J. Med. Chem. 83:650-656 (1988); Emmer, G. et al., J. Med. Chem. 28:278-281 (1985); Decker, H. et al., J. Gen Microbiol. 137:1805-1813 (1991); Delzer, J.et al., J. Antibiot. 37:80-82 (1984); all herein incorporated by reference).

In a preferred embodiment, the peptides of such conjugates will be N-.alpha.-acetylated, since such modification facilitates theuptake of peptide molecules.

In the method of reducing or preventing fungal growth, a composition containing at least one toxic analogue of enkephalin as an active ingredient are applied to a substrate or plant in an amount suitable to prevent or reduce fungal growth.

OPT1 Vectors

Vectors to allow the expression of OPT1 in plants comprise nucleic acid molecules comprising the coding sequence of OPT1, regulatory sequences suitable for use and functional in plants (such as promoters, enhancers, termination sequences and

the like), and may include selectable marker genes as is well known in the art (such as antibiotic resistance genes, and the like). Such vectors may be used to transform plant cells to provide expression of OPT1p in plants.

In one embodiment of the invention, the polynucleotides will be operably linked to regulatory sequences sufficient to permit the polynucleotide's transcription. Such polynucleotides may be incorporated into nucleic acid vectors that are sufficient to permit either the propagation or maintenance of the polynucleotide within a host cell. The nature of the regulatory elements will depend upon the host cell, and the desired manner of expressing the polynucleotide. Examples of suitable regulatory elements include constitutive or inducible prokaryotic promoters, such as the .lambda. pL or pR promoters, or other well-characterized promoters (e.g., lac, gal, trp, ara, hut, etc.). Other promoters which may be employed are the nos, ocs and CaMv promoters. Efficient plant promoters that may be used are over-producing plant promoters such as the small subunit (ss) of the ribulose 1, 5 biphosphate carboxylase from soybean (Berry-Lowe, et al., J. Molec. App. Gen. 1:483-498 (1982)) and the promoter of the chlorophyll a/b binding protein. These two promoters are known to be light induced in eukaryotic plant cells (see Genetic Engineering of Plants, An Agricultural Perspective," Cashmore, A. (ed), Plenum, N.Y., pp. 29-38 (1983); Coruzzi, G. et al., J. Biol. Chem. 258:1399 (1983); and Dunsmeier, P. et al., J. Molec. App. Gen. 2:285 (1983)). The 35S promoter is particularly preferred.

Preferred prokaryotic vectors include plasmids such as those capable of replication in E. coli such as, for example, pBR322,ColE1, pSC101, pACYC 184, .pi.VX. Such plasmids are, for example, disclosed by Maniatis, T., et al. (In:

Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1982)). Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: The Molecular Biology of the Bacilli, Academic Press, N.Y. (1982), pp. 307-329). Suitable Streptomyces plasmids include plJ101 (Kendall, K. J., et al., J. Bacteriol.169:4177-4183 (1987)), and Streptomyces bacteriophages such as .o slashed.C31 (Chater, K. F., et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John, J. F., et al. (Rev. Infect. Dis. 8:693-704 (1986)), and Izaki, K. (Jpn. J. Bacteriol. 33:729-742 (1978)).

As indicated, the invention particularly contemplates providing the polynucleotides of the present invention to plants, especially tobacco, coffee, wheat and other cereals, apple and other non-citrus fruit producers, and citrus fruit crops. Suitable plants include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonefla, Vigna, Citrus, Linum, Geranium, Manicot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, A tropa, Capsicum, Datura, Hyoscyamus, Lycopersion, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hemerocallis, Nemesia, Pelargonium, Panicurn, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browallia, Glycine, Lolium, Zea, Triticum, Sorghum, Ipomoea, Passiflora, Cyclamen, Malus, Prunus, Rosa, Rubus, Populus, Santalure, Allium, Lilium, Narcissus, Ananas, Arachis, Phaseolus, Pisum and Datura.

In one embodiment, OPT1 polynucleotide is provided without promoters or other regulatory elements, but under conditions sufficient to permit the polynucleotide

to recombine with and replace a region of the endogenous plant peptide transport gene. In an alternative embodiment, the polynucleotides will be administered to the plant operably linked to regulatory elements and/or vector elements.

Any of a variety of methods may be used to introduce the OPT1 polynucleotide of the present invention into a plant cell. The genetic material can be microinjected directly into the plant embryo cells or introduced by electroporation as described in Fromm et al.,"Expression of Genes Transformed into Monocot and Dicot Plant Cells by Electroporation," Proc. Nat'l. Acad. Sci. U.S.A.82:5824-28 (1985) or it can be introduced by direct precipitation using polyethylene glycol as described in Paszkowski et al.,EMBO J. 3:2717-22 (1984). In the case of monocotyledonous plants, pollen may be transformed with total DNA or an appropriate functional clone providing resistance, and the pollen then used to produce progeny by sexual reproduction.

The Ti plasmid of *Agrobacterium tumefaciens* provides a means for introducing DNA into plant cells (Caplan, A., et al., Science815-821 (1983); Schell, J. et al., Bio/Technology, April 1983, pp. 175-1980; Fraley, R. T., et al., Proc. Nat'l. Acad. Sci. U.S.A. 80:4803 (1983); (Hooykass, P. J. J. et al., In: Molecular Form and Function of the Plant Genome, Vlotan-Doltan, L. et al. (eds.), Plenum Press, N.Y., pp. 655-667 (1984); Horsch, R. B. et al., In: Current Communications in Molecular Biology, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., pp. 13-19 (1988); Horsch et al., Science 233:496-498 (1984); all herein incorporated by reference). As such, it provides a highly preferred method for introducing the polynucleotides of the present invention into plant cells

Ti plasmids contain two regions essential for the production of transformed cells. One of these, termed "transfer DNA" (TDNA), induces tumor formation. The other, termed "virulent region," is essential for the formation but not maintenance of tumors. It is possible to insert the polynucleotides of the present invention into the T DNA region without affecting its transfer function. By removing the tumor-causing genes so that they no longer interfere, the modified Ti plasmid can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate plant cell. The polynucleotides of the present invention are preferably inserted between the terminal sequences that flank the T-DNA.

A particularly useful Ti plasmid vector is pGV3850, a non-oncogenic derivative of the nopaline Ti plasmid C58 (Caplan, A., et al., Science 815-821 (1983)). This vector utilizes the natural transfer properties of the Ti plasmid. The internal T DNA genes that determine the undifferentiated crown gall phenotype have been deleted and are replaced by any commonly used cloning vehicle (such as pBR322). The cloning vehicle sequence contained between T DNA border regions serves as a region of homology for recombination to reintroduce foreign DNA cloned in a derivative of the same cloning vehicle. Any polynucleotide of the present invention cloned in such plasmid can thus be inserted into pGV3850 by a single recombination of the homologous sequences. Antibiotic resistance markers can be added to the plasmid to select for the recombination event. The presence of thenopaline synthase (nos) gene in pGV3850 facilitates the monitoring of the transformation.

The introduction of the Ti plasmid is typically accomplished by infecting a wounded leaf of the plant with *Agrobacterium tumefaciens* bacteria that contains the plasmid. Under appropriate growth conditions, a ring of calli forms around the wound(Hooykass, P. J. J. et al., In: Molecular Form and Function of the Plant Genome, Vlotan-Doltan, L. et al. (eds.), Plenum Press, N.Y., pp. 655-667 (1984)). The calli are then transferred to growth medium, allowed to form shoots, roots and develop further into plants.

The procedure can alternatively be performed in tissue culture. All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are recovered which contain the introduced polynucleotide. There is an increasing body of evidence that practically all plants can be regenerated from cultured cells or tissues, including but not limited to all major cereal crop species, sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables (Hooykass, P. J. J. et al., In: Molecular Form and Function of the Plant Genome, Vlotan-Doltan, L. et al. (eds.), Plenum Press, N.Y., pp. 655-667 (1984); Horsch, R. B. et al., In: Current Communications in Molecular Biology, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., pp. 13-19 (1988)). Methods for regenerating plants from cultural protoplasts are described by Evans et al. (Handbook of Plant Cell Culture 1:124-176; by Davey, M. R., In: Protoplasts1983--Lecture Proceedings, pp. 19-29, Birkhauser, Basel (1983)); Dale, P. J. (In: Protoplasts 1983--Lecture Proceedings, pp. 31-41, Birkhauser, Basel (1983)); Binding, H. (In: Plant Protoplasts, CRC Press, Boca Raton, pp. 21-37 (1985)) and Cooking, E. C. In:

Molecular Form and Function of the Plant Genome, Vlotan-Doltan, L. et al. (eds.), Plenum Press, N.Y., pp.27-32 (1984)).

Regeneration efficiency varies from species to species of plants, but generally a suspension of transformed protoplasts containing the introduced gene sequence is formed. Embryo formation can then be induced from the protoplast suspensions, to the stage of ripening and germination as natural embryos. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

Other systems, such as cauliflower mosaic virus, CaMV (Hohn, B., et al., In "Molecular Biology of Plant Tumors," Academic Press, New York, pp. 549-560; and Howell, U.S. Pat. No. 4,407,956) can also be used to introduce the OPT1 polynucleotide of the present invention into plant cells. In accordance with such methods, the entire CaMV viral DNA genome is inserted into apparent bacterial plasmid thus creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid is cleaved with restriction enzymes either at random or at unique sites in the viral portion of the recombinant plasmid for insertion of the polynucleotides of the present invention. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

After transformation of the plant cell or plant, the same may be selected by aid of an appropriate marker, such as antibiotic resistance, and then assessed to determine whether it contains the desired polynucleotide of the invention. The mature plants, grown from the transformed plant cells, can be selfed to produce an inbred plant whose seeds will contain the introduced polynucleotides of the present invention. These seeds can be grown to produce plants that exhibit any of a set of desired properties.

In one embodiment of the present invention, the exhibited property will be an increased facility to transport peptides, particularly, enkephalin. In this embodiment, the OPT1 polynucleotide of the invention are provided to the plant or plant cells along with transcriptional regulatory sequences, such that an overexpression of the plant's peptide transport gene occurs. Such plants are desirable in that their enhanced peptide transport system can be used to facilitate the up take of peptide-associated molecules.

The invention, therefore, also contemplates a method for growing plants, particularly crops such as those mentioned above, by providing said crops with the OPT1p encoding polynucleotide to allow for expression of OPT1p in the plant and providing the plant with a growth enhancement amount of an enkephalin to promote growth of the plant.

In all the embodiments of the invention, the polynucleotide of OPT1 that is suitable is at least the nucleotide sequence encoding the protein of SEQ ID NO:2.

EXPERIMENTS

Growth on Leu-Enkephalin-- An experiment was designed to determine whether members of the OPT family could transport leucine enkephalin (Leu-enkephalin;

YGGFL) to satisfy an auxotrophic requirement for leucine. For this study, a strain of *S. cerevisiae* auxotrophic for methionine and leucine (BY4730) along with the prototrophic parental strain (BY4700) were selected for use. Fig. 1 shows the growth of *S. cerevisiae* BY4730 expressing OPT family members. Cells were transformed with pDB20 (empty vector), pCaOPT1 (*C. albicans* OPT1) under its endogenous promoter, pADH194C (*S. cerevisiae* ORF 194C under the *S. cerevisiae* ADH promoter), and pADHOPT1 (*S. cerevisiae* OPT1 under the *S. cerevisiae* ADH promoter). Cells were spotted onto proline medium supplemented with various sources of leucine, as indicated on the figure, to meet auxotrophic requirements and were grown for 72 h at 30 °C. *S. cerevisiae* BY4730 transformed with the vector (pDB20) and transformants expressing three members of the OPT family were able to use either leucine or leucyl-leucine for growth as shown in Fig. 1. In contrast, only cells transformed with YJL212C, expressing OPT1 (pADHOPT1), could grow on Leu-enkephalin as a sole source of leucine. The parental strain BY4700 transformed with an empty vector (pDB20) or three members of the OPT family (pCaOPT1, pADH194C, or pADHOPT1) grew well in the presence of Leu-enkephalin at all concentrations (10-1000 μM), indicating this peptide was not toxic. Growth on Leu-enkephalin in cells expressing OPT1 was concentration-dependent, with the most robust growth seen at the highest concentrations. In a similar experiment, it was determined that cells expressing OPT1 could grow on methionine enkephalin (Met-enkephalin) as a sole source of methionine.

Transport of Radiolabeled Leu-Enkephalin-- To further explore the possibility that Leu-enkephalin transport was carrier-mediated, transport was measured directly using radiolabeled Leu-enkephalin ([^{3}H]YGGFL). Fig. 2 shows uptake of [^{3}H]Leu-enkephalin by *S. cerevisiae* BY4730 transformants. Part A shows uptake versus time at 30 °C (●) or 4 °C (○) for cells transformed with pADHOPT1 and at 30°C for the empty vector pDB20 (×). The inset in part A shows Leu-enkephalin uptake at 30°C versus concentration of Leu-enkephalin for cells transformed with pADHOPT1. Part B shows uptake versus pH for cells transformed with pADHOPT1.

Leu-enkephalin was transported into cells expressing OPT1 (Fig. 2A) in a time- and temperature-dependent manner. In contrast, cells transformed with the vector pDB20 did not accumulate enkephalin. The uptake of Leu-enkephalin was pH-dependent. Transport of the substrate was highest at pH 5.5 and declined sharply as the proton concentration was raised or lowered (Fig. 2B). This pH optimum is similar to those reported for the eukaryotic di- and tripeptide transport systems, as well as that for peptide transport in the prokaryote *Lactococcus lactis*. Treatment of cells with the metabolic uncouplers 2,4-dinitrophenol, CCCP, or sodium azide, all of which deplete intracellular ATP and collapse the proton gradient, or treatment with the sulfhydryl reagent pCMBS substantially reduced enkephalin uptake (Table I). These data are consistent with a carrier-mediated uptake system for Leu-enkephalin encoded by OPT1.

Table I

Leu-enkephalin uptake in the presence of various compounds

The uptake of Leu-enkephalin (250 μM) was measured over a 12-min time course in the presence of the compounds indicated. Each measurement was completed a minimum of four times. The results were normalized to uptake after 12 min of incubation measured in the absence of any other compound (none, 100%) and are reported as mean ± standard deviation.

Compound	Percent of control
None	100%
Leucine Enkephalin (YGGFL)[a]	12 ± 1%
Methionine Enkephalin (YGGFM)[a]	25 ± 4%
Tyrosine [a]	95 ± 12%
Leu-Leu [a]	97 ± 12%
Gly-Gly-Phe [a]	99 ± 5%
Gly-Gly-Phe-Leu [a]	41 ± 8%
Lys-Leu-Gly-Leu [a]	31 ± 14%
MIF-1 (PLG-NH_2)[a]	95 ± 7%
Tyr-MIF-1 (YPLG-NH_2)[a]	78 ± 9%
Tyr-Gly-Gly-Phe-Leu-NH_2 [a]	71 ± 5%
DPDPE (Y-D-Pen-GF-D-Pen)[a]	69 ± 11%
DADLE (Y-D-AGF-D-L)[a]	58 ± 5%
Sodium Azide[b]	21 ± 2%
2,4-Dinitrophenol[b]	17 ± 2%
CCCP[b]	38 ± 6%
pCMBS[b]	55 ± 5%

[a] All competitors were at a final concentration of 2.5 mM and added simultaneously with [^{3}H]Leu-enkephalin in the uptake medium.
[b] Cells were pre-incubated with sodium azide (1 mM), CCCP (0.1 mM) 2,4-dinitrophenol (1 mM), or pCMBS (0.2 mM) for 30 min prior to addition of the uptake medium.

Table I shows Leu-enkephalin uptake in the presence of various compounds. The uptake of Leu-enkephalin (250 μM) was measured over a 12-min time course in the presence of the compounds indicated. Each measurement was completed a minimum of four times. The results were normalized to uptake after 12 min of incubation measured in the absence of any other compound (none, 100%) and are reported as mean ± standard deviation.

Fig. 3 shows a chromatographic analysis of Leu-enkephalin. Arrows indicate the RF values for tyrosine and intact Leu-enkephalin. Part A shows an analysis of uptake assay medium after 2-min incubation with BY4730 transformed with pADHOPT1. Similar analysis of medium prior to incubation with cells produced identical results. Part B shows an analysis of material extracted from cells after 12-min incubation interval.

As shown in Figure 3, the rate of Leu-enkephalin uptake remained relatively constant over a 12-min time course, suggesting that the opioid does not remain intact upon entering the cell. Chromatographic analysis of radiolabeled material extracted from cells indicated that the enkephalin was degraded, with virtually all radioactivity associated with free tyrosine. In contrast, analysis of an aliquot of medium from which cells were removed after 12 min of incubation at 30 °C revealed that no extracellular hydrolysis of the peptide had occurred. All radioactivity was still associated with intact Leu-enkephalin. If it is assumed that translocation of the substrate, rather than its hydrolysis, is rate-limiting, then an apparent Km for transport can be determined by measuring the rate of transport as a function of substrate concentration. Transformation of these data give an apparent Km of 310 μM for the uptake of Leu-enkephalin by transporter (Fig. 2A, inset)

From Table I, the transport protein encoded by OPT1 has a strong preference for both Leu-enkephalin and Met-enkephalin and does not appear to transport amino acids or di- or tripeptides. Accumulation of Leu-enkephalin was not affected by the presence of tyrosine or the di- and tripeptides tested, suggesting that the OPT1 protein does not recognize these compounds. The uptake of radiolabeled Leu-enkephalin decreased by 75-88% in the presence of a 10-fold molar excess of Met-enkephalin or Leu-enkephalin, respectively. The tetrapeptide Lys-Leu-Gly-Leu (KLGL), a known substrate for other oligopeptide transporters was also an effective competitor. The amidated form of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu-NH_2) showed weak inhibition of enkephalin uptake in yeast.

Inhibition of Uptake by Enkephalin Analogs-- The nonmetabolized pentapeptide enkephalin analogues DADLE and DPDPE were somewhat effective competitors, blocking 30-40% of the uptake (Table I). The amidated tetrapeptide Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2), a substrate for the previously described PTS-1 whole brain Met-enkephalin transport system was a poor competitor, reducing Leu-enkephalin uptake by only 20%. The tripeptide MIF-1 did not cause a significant reduction in the uptake of Leu-enkephalin, further emphasizing the preference of the OPT1 system for tetra- and pentapeptides.

Fig. 4 shows the effects of naloxone and naltrexone on the uptake of [^{3}H]Leu-enkephalin and [^{3}H]leucyl-leucine. Part A shows the uptake of Leu-enkephalin (250 μM) as measured over a 12-min time course in the presence of naloxone (black bars) or naltrexone (shaded bars) at the concentrations indicated. The results were normalized to uptake of Leu-enkephalin (control, open bar) measured in the absence of either

compound and are reported as mean ± standard deviation. Part B shows the uptake of leucyl-leucine (160 μM) as measured over a 12-min time course in the presence and absence of naloxone (black bar) or naltrexone (shaded bar) at the concentrations indicated. Results were normalized to control and reported as described for part A.

Naloxone and naltrexone antagonize the binding of enkephalin to the opioid receptor. It was found that these compounds also inhibit the transport of Leu-enkephalin across Opt1p (Fig. 4A). In a similar experiment, the presence of these compounds did not inhibit the transport of leucyl-leucine, a substrate for the di- and tripeptide transport system Ptr2p (Fig. 4B).

We have discovered a function of the previously unknown open reading frame YJL212C in the yeast *S. cerevisiae*. This gene is *OPT1* (SEQ ID NO. 1). The protein encoded by *OPT1* (SEQ ID NO. 2) consists of 799 amino acids, and based on the amino acid sequence the predicted protein structure suggests an integral membrane protein containing 12-14 putative membrane-spanning domains. In addition, the protein contains several motifs unique to the OPT family, the largest of which consists of 10 invariable residues (SPYXEVRXXVXXXDDP) located before the first hydrophobic domain. OPT1, like other members of the OPT family, encodes a functional oligopeptide transporter.

Because Opt1p exhibited all the molecular characteristics of an OPT family member, it was hypothesized that this protein was an oligopeptide transporter, even though it was known that *S. cerevisiae* could not utilize any tetra- or pentapeptides tested to date to satisfy auxotrophic requirements under routine growth conditions. To see activity of Opt1, it was necessary to express OPT1 under the control of the ADH

promoter, a strong, constitutive promoter which would presumably result in high expression of the gene product. Northern blot analysis confirmed that OPT1 was not expressed at detectable levels under routine conditions of logarithmic growth. These results were independently confirmed by serial analysis of gene expression (SAGE) which revealed that OPT1 is only expressed at a low level (~1 copy per cell) following nocodazole arrest in the G2/M phase of the cell cycle. Additional analysis of sporulating yeast cells by DNA microarray analysis indicated that OPT1 was expressed during the late stages of sporulation. In light of these observations, OPT1gene expression must be ectopically induced under the control of a heterologous promoter to enable study of Opt1p function in log phase cells.

The product of OPT1 is the oligopeptide transporter Opt1p, which translocates pentapeptides, including both Met- and Leu-enkephalin. In BY4730, a strain of *S. cerevisiae* auxotrophic for leucine and methionine, only cells expressing OPT1 could grow on Leu-enkephalin in the absence of exogenous leucine. This indicates that enkephalins are transported intact into the cell and then hydrolyzed. If oligopeptides were hydrolyzed by an extracellular protease prior to transport, then the isogenic control strain (BY4730 transformed with the empty vector pDB20), as well as yeast cells transformed with plasmids encoding other OPT family members (CaOPT1, YPR194C) should be able to utilize the hydrolysis products for growth. This was not the case. Chromatographic analysis supports conclusion. No evidence for degraded forms of Leu-enkephalin could be found in the extracellular medium. In addition, a large body of work exists which demonstrates that di- and tripeptides enter the cell intact and are then rapidly hydrolyzed by intracellular peptidases.

Transport of Leu-enkephalin is pH- and temperature-dependent, suggesting that this is a proton-coupled, energy-dependent process. These observations are supported by the sensitivity of the transporter to agents which disrupt the proton gradient or deplete intracellular ATP. Utilization of the transmembrane proton gradient to energize active transport has been demonstrated for the PTR family of di- and tripeptide transporters. Uptake of radiolabeled Leu-enkephalin was inhibited in the presence of excess unlabeled Met- or Leu-enkephalin; amidated Leu-enkephalin was an ineffective competitor. Tyr-MIF-1 is an amidated tetrapeptide with opiate and anti-opiate activity. This peptide is a substrate for the previously described blood-brain barrier PTS-1 enkephalin transport activity but, like the amidated form of authentic Leu-enkephalin, was not an effective competitor for yeast Opt1p. This observation is consistent with the need for a free carboxyl terminus for substrate recognition by Opt1p. Tetrapeptides were effective inhibitors, with Lys-Leu-Gly-Leu and des-Tyr1 Leu-enkephalin (Gly-Gly-Phe-Leu) eliminating over 50% of radiolabeled enkephalin accumulation, suggesting that an amino-terminal tyrosine is not essential for substrate recognition. Neither the tripeptide enkephalin fragment Gly-Gly-Phe nor the dipeptide Leu-Leu could inhibit uptake, indicating that this system is distinct from Ptr2p and is selective for tetra- and pentapeptides. These data show that intact oligopeptides are gaining access to the cell via a carrier-mediated process, and the discrete carrier is the gene product of OPT1. If enkephalins were entering by a nonspecific mechanism such as simple diffusion or endocytosis, then all strains, not just those expressing OPT1, should be able to utilize this substrate.

Several enkephalin antagonists were assayed in this study for their effect on enkephalin transport across Opt1p. DADLE and DPDPE are enzymatically stable delta opioid receptor antagonists that are pentapeptide mimetics. Previous reports indicated that DPDPE gained access to the brain by a saturable, carrier-mediated mechanism in the blood-brain barrier, which has yet to be defined. Interestingly, transport of DPDPE was not inhibited by Leu-enkephalin in those studies, suggesting either the existence of separate transport systems or a common system with different affinities for these two substrates. A recent report suggests that DPDPE crosses the blood-brain barrier by a phenylarsine oxide-sensitive pathway, suggesting a role for a saturable endocytic mechanism in the *in vitro* and *in situ* models studied. It was found that DPDPE and DADLE were weak competitors for Leu-enkephalin transport, indicating that Opt1p interacts with the stable antagonists with differential affinities compared with authentic Leu-enkephalin.

Naloxone and naltrexone are synthetic opioid receptor antagonists classically used to reverse the effects of opiate overdose. Naltrexone is also used clinically in the treatment of alcoholism. Despite the fact that these compounds are similar in structure to morphine, rather than resembling a peptide, they were effective competitors for Leu-enkephalin transport. The effect appears to be specific for the Opt1p transporter because the presence of the morphine analogs did not influence the activity of the unrelated di- and tripeptide transporter Ptr2p. The nature of the inhibition of Leu-enkephalin transport by naloxone and naltrexone is currently under investigation. Specifically, it would be of interest to determine whether these compounds are substrates for transport or are nonsubstrate competitors for Opt1p.

There is increasing evidence that opioids and their analogues enter the central nervous system by carrier-mediated transport across the blood-brain barrier. Evidence also exists to suggest that the clearance of the enkephalin analogue DPDPE occurs by saturable efflux from the brain and systemic elimination of intact DPDPE via biliary excretion. Furthermore, it is possible that neuronal re-uptake systems exist for enkephalin similar to the well studied transport systems for neurotransmitters such as serotonin and γ-aminobutyric acid. Previously, none of the putative transporters for enkephalin have been cloned or characterized at a molecular level. The present invention presents the first evidence for a genetically defined eukaryotic transport protein, Opt1p, which recognizes and translocates both Met- and Leu-enkephalin into an intact eukaryotic cell. The identification of this transporter in *Saccharomyces* may facilitate the discovery of mammalian homologues, thus providing greater insight into the process of pain and its mediation. These mammalian homologues may aid in transporting opiates across the blood-brain barrier, and mediation of the homologues could allow pain mediation. Similarly, the homologues may be helpful in substance abuse treatment or in finding competitors for opiate transport mechanism to aid such treatment.

EXAMPLES

Strains, Media, and Vectors-- BY4700 (Mata ura3Δ0) and BY4730 (Mata leu2Δ0 met15Δ0 ura3Δ0) were grown routinely on YEPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Strains transformed with a plasmid were cultured on minimal medium lacking uracil (0.67% Difco yeast nitrogen base with ammonium sulfate, without amino acids, 2% glucose, 0.2% casamino acids). For growth assays, cells were inoculated into medium lacking uracil and ammonium sulfate (0.67% Difco yeast

nitrogen base without amino acids and ammonium sulfate, 2% glucose) supplemented with 0.1% proline as a nitrogen source, 228 μM leucine, and 191 μM methionine (proline medium). The plasmids pADH212C and pADH194C were created by polymerase chain reaction amplification of the appropriate ORFs (YJL212C and YPR194C, respectively) and cloning the resultant products into the URA3/2μ-based vector pDB20 such that the genes were under the control of an ADH promoter. The plasmid pCaOPT1 consists of a 3.8-kilobase genomic fragment from *C. albicans* which contains the CaOPT1 gene cloned into pRS202, a URA3/2 μ-based plasmid. Plasmids were transformed into yeast by the method of Geitz, and transformants were selected by growth on minimal medium lacking uracil.

Growth and Uptake Assays-- Transformed cells were grown overnight to mid-exponential phase in proline medium. For growth assays, cells were harvested, washed, and adjusted to a final concentration of 2×10^7 cells/ml in water. Five microliters ($= 1 \times 10^5$ cells) of each sample was spotted onto proline medium plus 2% agar, supplemented with amino acids or peptides, as indicated in the text and Fig. 1. Plates were incubated at 30 °C for 72 h and observed for growth. For uptake assays, cells were harvested and washed with 2% glucose and adjusted to a final concentration of 2×10^8 cells/ml. The uptake assay was initiated by combining equal volumes of pre-warmed (30 °C) cells and 2× uptake assay mixture (2% glucose, 20 mM sodium citrate/potassium phosphate, pH 5.5, 500 μM Leu-enkephalin (Sigma), 1 μCi/ml [^{3}H]leucine enkephalin (50 Ci/mmol, American Radiolabeled Chemicals), and incubating at 30 °C. For determination of leucyl-leucine accumulation, 320 μM L-leucyl-L-[^{3}H]leucine (16 mM, 10 mCi/mmol) was used in place of Leu-enkephalin. L-Leucyl-L-[^{3}H]leucine was synthesized by standard solution-phase

techniques. For assays done in the presence of competitors, the 2× uptake assay mixture was supplemented with competitor (2× final concentration) prior to combining with the cells. A concentrated stock of carbomyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma) was prepared in Me_2SO; naloxone and naltrexone (Sigma) were dissolved in methanol. The compounds were diluted such that the solvent was present at a final concentration of 5% in the uptake medium. All other compounds were dissolved in either water or sodium citrate/potassium phosphate buffer (pH 5.5). At the appropriate time, aliquots (90 µl) were removed and washed by vacuum filtration with 4 × 1 ml ice cold water onto a membrane filter (HAWP, Millipore). The membranes were counted by liquid scintillation spectrometry, and results were reported as nmol/mg dry weight. Data points reflect the mean and standard deviation of a minimum of four independent measurements.

Chromatography-- Cells were incubated with uptake medium for 12 min, harvested, and washed four times with ice-cold water. The cell pellet was extracted by boiling in 50% methanol. The methanol extracts, along with control samples, were spotted onto silica plates and developed by ascending chromatography using butanol:glacial acetic acid:water solvent system (9:1:2.5). The chromatograms were sprayed with ninhydrin (0.1% in 95% ethanol) to visualize the nonradioactive standards. Lanes containing radioactive samples were scraped in 0.8-cm intervals and counted for retained radioactivity.

CLAIMS

We claim:

1. A method for obtaining mammalian enkephalin transport proteins comprising deleting the *OPT1* gene from a strain ofyeast, transforming said strain with a library of mammalian genes cloned into a suitable yeast expression plasmid, thereby forming transformed yeast, growing said transformed yeast on a medium with an appropriate amount of an enkephalin, selecting transformed yeast based on an ability of said transformed yeast to transport said enkephalin.

2. The method of claim 1 wherein said yeast strain is selected from the group consisting of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.*

3. The method of claim 1 wherein said enkephalin in Leu-enkephalin.

4. An antifungal composition comprising a toxic derivative of enkephalin as an active ingredient in a sufficient amount to prevent or reduce fungal growth.

5. The antifungal composition of claim 4 wherein said toxic derivative of enkephalin comprises a toxic molecule conjugated to an enkephalin.

6. The antifungal composition of claim 5 wherein said toxic molecule is selected from the group consisting of N3-(4-methoxyfumaroyl)-L-2,3 diaminopropanoic acid, 5-fluororotic acid, dideoxynucleotides, mutagenic nucleotide analogues, mutagenic nucleoside analogues, and toxic amino acids.

7. The antifungal composition of claim 6 wherein said toxic amino acids are selected from the group consisting of oxalysine, fluorophenylalanine, ethionine and unusual D-amino acids.

8. A method of reducing or preventing fungal growth comprising applying an effective amount of an antifungal composition to a substrate wherein said antifungal compound comprises a toxic derivative of enkephalin as an active ingredient in a sufficient amount to prevent or reduce fungal growth.

9. The method of claim 8 wherein said substrate is a plant.

10. A vector for transformation of plant cells comprising, operably joined, a promoter functional in plants, regulatory sequences for transcription and translation functional in plants, and a nucleic acid molecule encoding the protein of SEQ ID NO:2.

11. Transformed plant cells comprising plant cells harboring the vector of claim 10.

12. A method for cultivating plant material comprising transforming plant material with the vector of claim 10, and providing a sufficient amount of an enkephalin to said plant material to enhance plant and plant part growth.

13. The method of claim 12 wherein said plant material is a crop plant selected from the genera selected from the group consisting of Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonefla, Vigna, Citrus, Linum, Geranium, Manicot, Daucus, Arabidopsis, Brassica, Raphanus,

Sinapis, A tropa, Capsicum, Datura, Hyoscyamus, Lycopersion, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hemerocallis, Nemesia, Pelargonium, Panicurn, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browallia, Glycine, Lolium, Zea, Triticum, Sorghum, Ipomoea, Passiflora, Cyclamen, Malus, Prunus, Rosa, Rubus, Populus, Santalure, Allium, Lilium, Narcissus, Ananas, Arachis, Phaseolus, Pisum and Datura..

14. The method of claim 12 wherein said plant part growth comprises growth of fruit of said plant.

15. A method for obtaining mammalian enkephalin transport proteins comprising transforming a strain of yeast with a library of mammalian genes cloned into a suitable yeast expression plasmid, thereby forming transformed yeast, growing said transformed yeast on a medium with an appropriate amount of an enkephalin, selecting transformed yeast based on an ability of said transformed yeast to transport said enkephalin.

16. The method of claim 15 wherein said yeast strain is selected from the group consisting of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

17. The method of claim 15 wherein said enkephalin is Met- or Leu-enkephalin.

18. The method of claim 15 wherein said yeast expression plasmid comprises a strong promoter for expression in yeast that drives expression of said mammalian gene cloned into said plasmid.

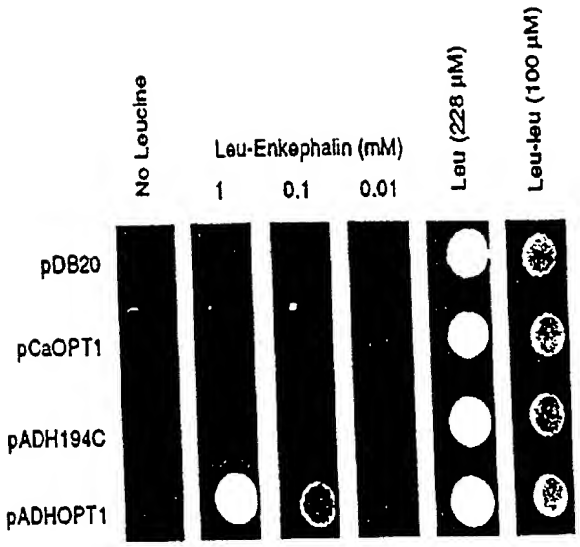
No Leucine
Leu-Enkephalin (mM)
1
0.1
0.01
Leu (228 µM)
Leu-leu (100 µM)
pDB20
pCaOPT1
pADH194C
pADHOPT1

FIG. 1

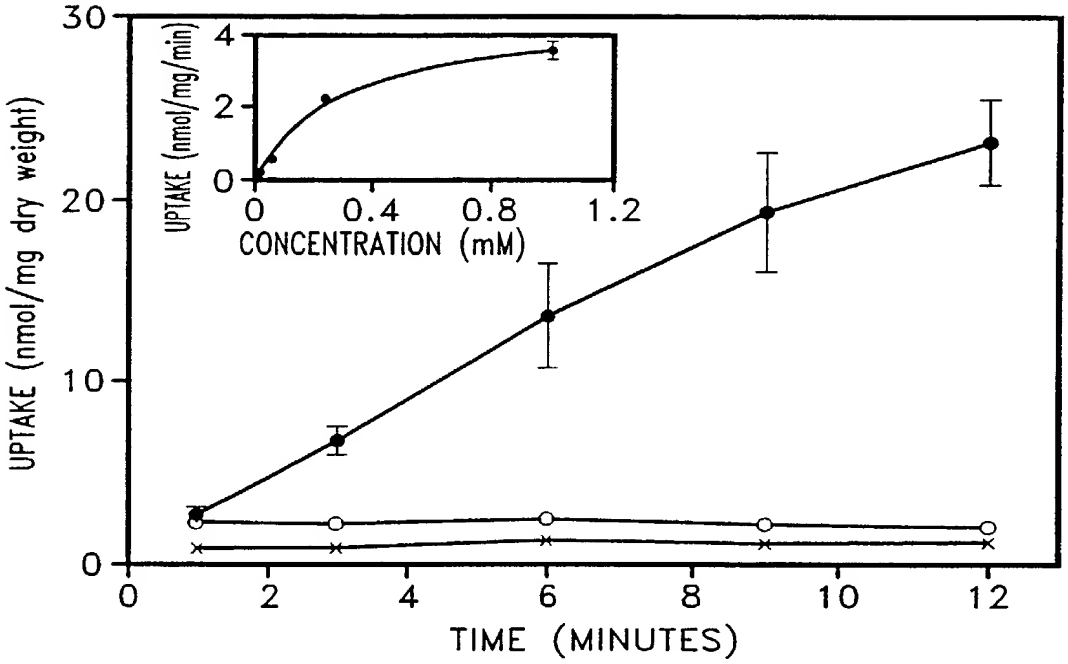
UPTAKE (nmol/mg dry weight)
30
20
10
0
0
2
4
6
8
10
12
TIME (MINUTES)
UPTAKE (nmol/mg/min)
4
2
0
0
0.4
0.8
1.2
CONCENTRATION (mM)

FIG. 2A

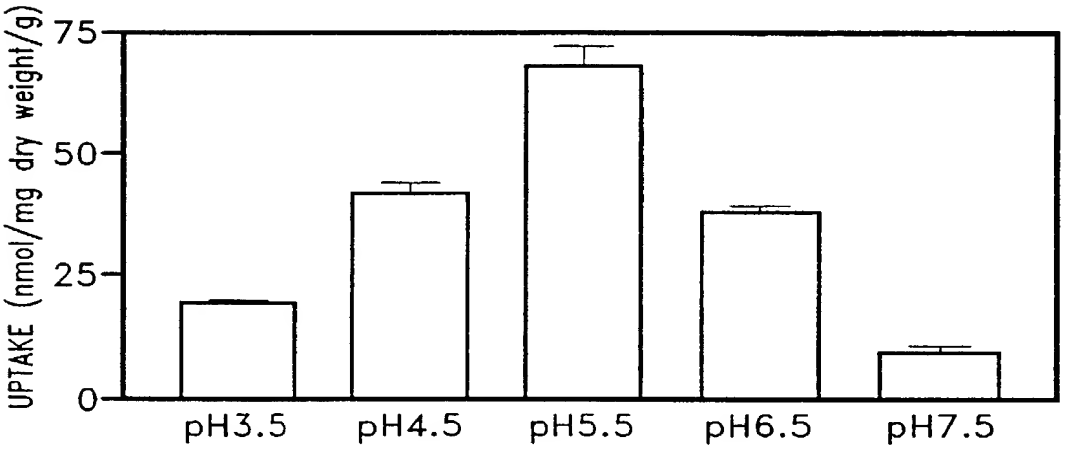
UPTAKE (nmol/mg dry weight/g)
75
50
25
0
pH3.5
pH4.5
pH5.5
pH6.5
pH7.5

FIG. 2B

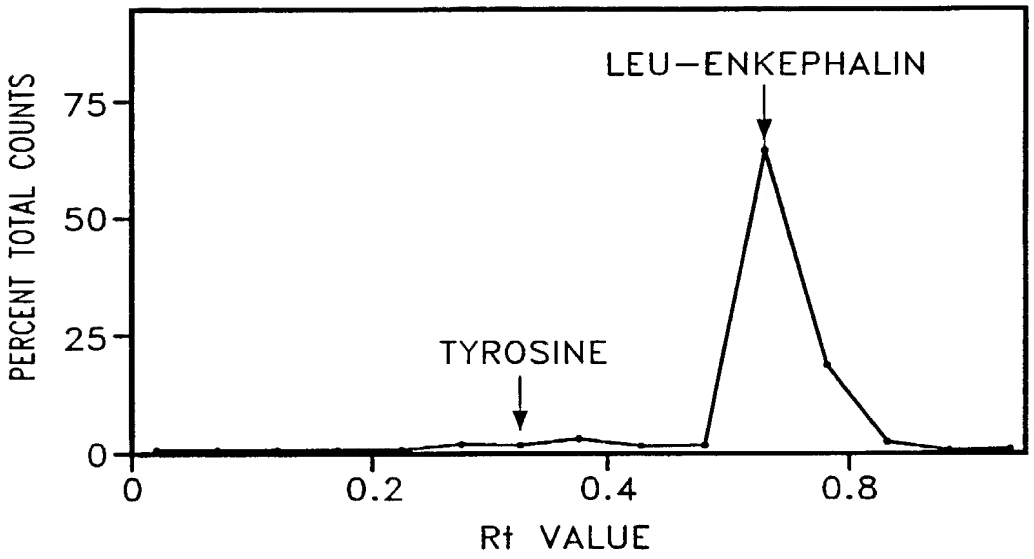
LEU—ENKEPHALIN
TYROSINE
PERCENT TOTAL COUNTS
75
50
25
0
0
0.2
0.4
0.8
Rt VALUE

FIG. 3A

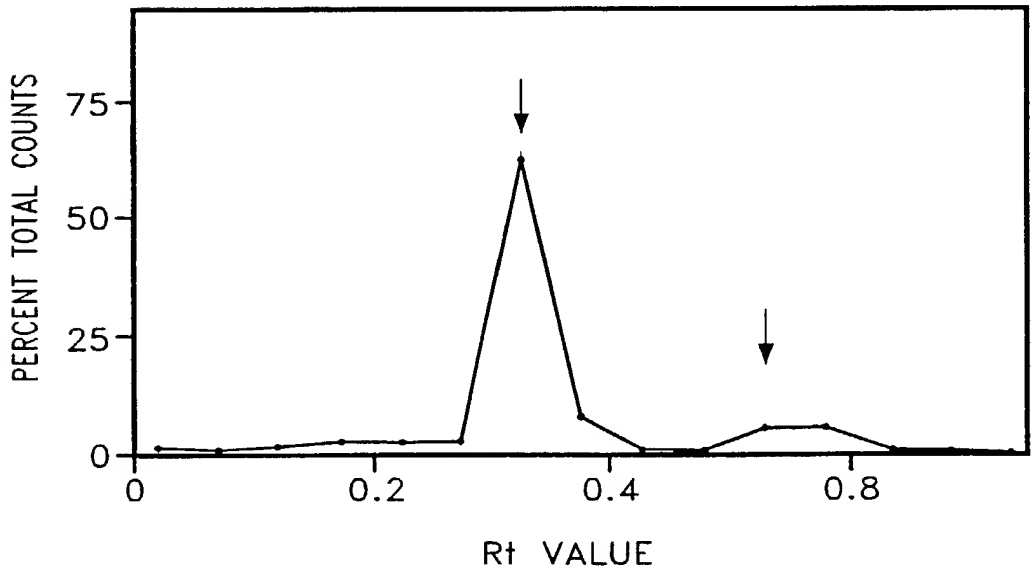
PERCENT TOTAL COUNTS
75
50
25
0
0
0.2
0.4
0.8
Rt VALUE

FIG. 3B

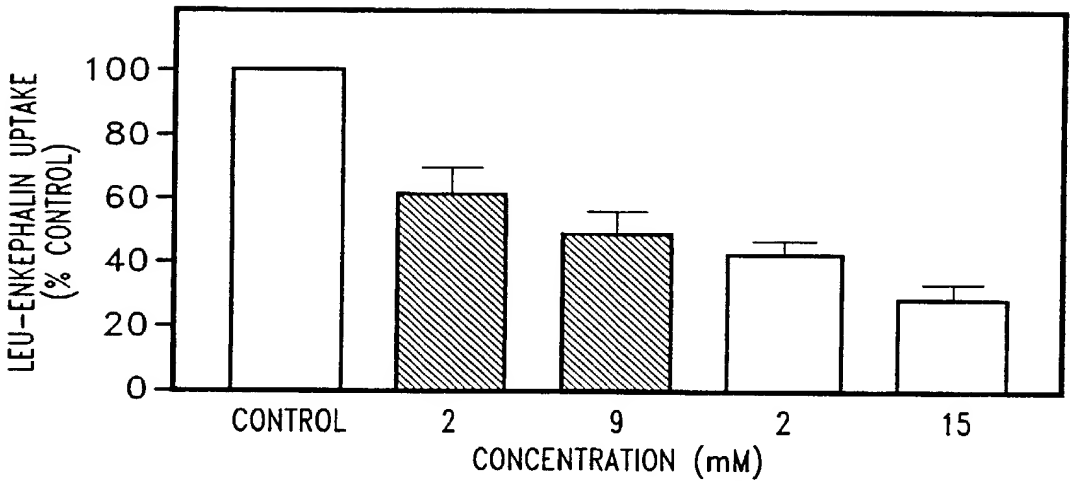
LEU-ENKEPHALIN UPTAKE
(% CONTROL)
100
80
60
40
20
0
CONTROL
2
9
2
15
CONCENTRATION (mM)

FIG. 4A

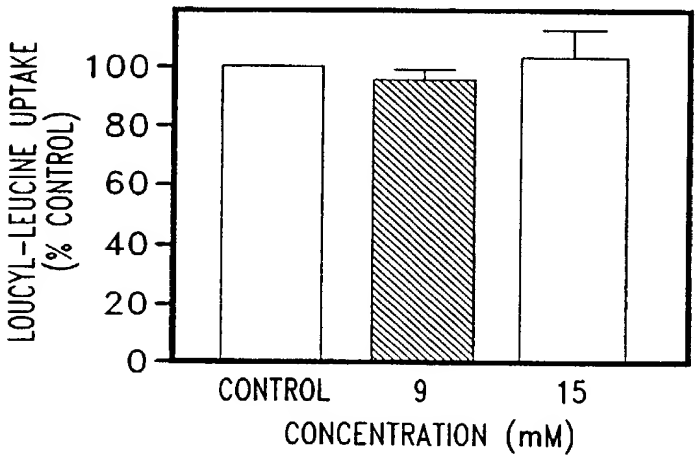
LOUCYL-LEUCINE UPTAKE
(% CONTROL)
100
80
60
40
20
0
CONTROL
9
15
CONCENTRATION (mM)

FIG. 4B

MVGSLEVSKPPEHKVESKIVIADEEEDENDSPIEEVRLTVPITDDPSLPVLTFRTWFLGMVSCVVLAFV
NNFFGYRSNPLTVSSVVAQIITLPLGKLMATTLPTTKLRLPGTNWSCSLNPGPFNMKEHVLITIFANTGA
GGAYATSILTIVKAFYHRNLNPAAAMLLVQTTQLLGYGWAGMFRKYLVDSPYMWWPANLVQVSLFRALHE
KEEKREGKQTKLRFFLIVFFLSFTYYIVPGYLFPSISYLSFVCWIWTRSVTAQQIGSGLHGLGIGSFGLD
WSTVAGFLGSPLAVPFFAIANSFGGFIIFFYIILPIFYWSNAYEAKKFPFYTSHPFDHTGQRYNTTRILN
QKTFNIDLPAYESYSKLYLSILFALIYGLSFGTLTATISHVALFDGKFIWELWKKATLTTKDKFGDVHTR
LMKKNYKEVPQWFVAVLAASFVLALYACEGFGKQLQLPWWGLLLACAIAFTFTLPIGVILATTNQRMGL
NVISELIIGFLYPGKPLANVAFKTYGSVSIAQALYFVGDFKLGHYMKIPPRSMFIVQLVATIVASTVSFG
TTWWLLSSVENICNTDMLPKSSPWTCPGDVVFYNASIIWGIIGPGRMFTSKGIYPGMNWFFLIGFLAPVP
VWFFARKFPEKKWIHQIHIPLIFSGANVMPMAKAVHYWSWFAVGIVFNYYIFRRYKGWWARHNYIILSAAL
DAGTAVMGVLIYFALQNNNISLPDWWGNENTDHCPLANCPTEKGIVAKGCPVF

FIG. 5

MDAEKATDKTNVHLSSDHERCPVEEVALVVPETDDPSLPVMTFRACHSPSQPSLMQIAGLPIGKFMARTL
PTTSHNLLGWSFSLNPGPFNIKEHVIITIFANCGVAYGGGDAYSIGAITVMKAYYKQSLSFICGLFIVLT
TQILGYGWAGILRRYLVDPVDMWWPSNLAQVSLFRALHEKENKSKGLTRMKFFLVALGASFIYYALPGYL
FPILTFSSWVCWAWPNSITAQQVGSGYHGLGVGAFTLDWAGISAYHGSPLVAPWSSILNVGVGFIMFIYI
IVPVCYWKFNTFDARKFPISSNQLFTTSGQKYDTTKILTPQFDLDIGAYNNYGKLYLSPLFALSIGSGFA
RFTATLTHVALFNGRDIWKQTWSAVNTTKLDIHGKLMQSYKKVPEWWFYILLAGSVAMSLLMSFVWKESV
QLPWWGMLFAFALAFIVTLPIGVIQATTNQQPGYDIIGQFIIGYILPGKPIANLIFKIYGRISTVHALSF
LADLKLGHYMKIPPPCMYTAQLVGTVVAGVVNLGVAWWMLESIQDICDIEGDHPNSPWTCPKYRVTFDAS
VIWGLIGPRRLFGPGGMYRNLVGFFLIGAVLPVPRVGAEQDLPKQEVDPSHQHSSYLLRLCRDASSHSNQ
HCQLVGHRNHLQLLCVQLPQEMVAEVQLRTLCSARCRDRVHGGALVLRPAECWTRPQMVGH

FIG. 6

MEESEQVLPLLTNPKDLTNPSYASSSSSSSEPRDETEDLLLPISDENEEEENSPIRQVALTVPTTDDP
SLPVLTFRMWVLGTLSCILLSFLNQFFWYRTEPLTISAISAQIAVVPLGRLMAAKITDRVFFQGSKWQFT
LNPGPFNVKEHVLITIFANAGAGSVYAIHVVTVVKAFYMKNITFFVSFIVIVTTQVLGFGWAGIFRKYLV
EPAAMWWPANLVQVSLFRALHEKEERTKGGLTRTQFFVIAFVCSFAYYVFPGYLFQIMTSLSWVCWFFPS
SVMAQQIGSGLHGLGVGAIGLDWSTISSYLGSPLASPWFATANVGVGFVLVIYVLVPICYWLDVYKAKTF
PIFSSSLFSSQGSKYNITSIIDSNFHLDLPAYERQGPLYLCTFFAISYGVGFAALSATIMHVALFHGREI
WEQSKESFKEKKLDVHARLMQRYKQVPEWWFWCILVTNVGATIFACEYYNDQLQLPWWGVLLACTVAIIF
TLPIGIITAITNQAPGLNIITEYIIGYIYPGYPVANMCFKVYGYISMQQAITFLQDFKLGHYMKIPPRTM
FMAQIVGTLISCFVYLTTAWWLMETIPNICDSVTNSVWTCPSDKVFYDASVIWGLIGPRRIFGDLGLYKS
VNWFFLVGAIAPILVWLASRMFPRQEWIKLINMPVLISATSSMPPATAVNYTTWVLAGFLSGFVVFRYRP
NLWQRYNYVLSGALDAGLAFMGVLLYMCLGLENVSLDWWGNELDGCPLASCPTAPGIIVEGCPLYT

FIG. 7

MGEIATEFTPVMDDDDRCVVPEVELTVPKTDDSTLPVLTFRMWVLGIGACIVLSFINQFFWYRTMPLSI
TGISAQIAVVPLGHLMARVLPTKRFLEGTRFQFTLNPGAFNVKEHVLITIFANSGAGSVYATHILSAIKL
YYKRSLPFLPAFLVMITTQILGFGWAGLFRKHLVEPGEMWWPSNLVQVSLFGALHEKEKKSRGGMSRTQF
FLIVLVASFAYYIFPGYLFTMLTSISWVCWLNPKSILVNQLGSGEHGLGIGSIGFDWVTISAYLGSPLAS
PLFASVNVAIGFVLVMYIVTPVCYWLNIYDAKTFPIFSSQLFMGNGSRYDVLSIIDSKFHLDRVVYSRTG
SINMSTFFAVTYGLGFATLSATIVHVLVFNGSDLWKQTRGAFQKNKKMDIHTRIMKKNYREVPLWWFLVI
LLLNIALIMFISVHYNATVQLPWWGVLLACAIAISFTPLIGVIAATTNQAPGLNIITEYVIGYIYPERPV
ANMCFKVYGYISMTQALTFISDFKLGHYMKIPPRSMFMAQVAGTLVAVVVYTGTAWWLMEEIPHLCDTSL
LPSDSQWTCPMDRVFFDASVIWGLVGPRRVFGDLGEYSNVNWFFLVGAIAPLLVWLATKMFPAQTWIAKI
HIPVLVGATAMMPPATAVNFTSWLIVAFIFGHFIFKYRRVWWTKYNYVVLSGGLDAGSAFMTILLFLALGR
KGIEVQWWGNSGDRDTCPLASCPTAKGVVVKGCPVF

FIG. 8

MAAIELHKPEINADDDDDESPVEQVRLTVSNHDDPSLPVWTFRMWFLGLLSCILLSFLNTFFGYRTQPLM
ITMISVQVVTLPLGKLMARVLPETKYKIGSWEFSFNPGPFNVKEHVLISMFANAGAGFGSGTAYAVGIVD
IIMAFYKRKISFLASWILVITTQDNARMSRGKFFVIAFVCSFAWYIFPAYLFLTLSSISWVCWAFPKSIT
AQQLGSGMSGLGIGAFALDWSVIASYLGSPLVTPFFAIVNVLVGYVLVMYMVIPISYWGMNVYEANKFPI
FSSDLFDKQGQLYNISTIVNNKFELDMENYQQQGRVYLSTFFAISYGIGFAAIVSTLTHVALFNGKGIWQ
QVRASTKAKMDIHTRLMKKYKDIPGWWFYSLLAISLVLSLVLCIFMKDEIQMPWWGLLLASFMALTFTVP
VSIITATTNQTPGLNIITEYLMGVLLPGRPIANVCFKTYGYISMSQAISFLNDFKLGHYMKIPPRSMFLV
QFIGTVIAGTVNISVAWYLLTSVENICQKELLPPNSPWTCPSDRVFFDASVIWGLVGPKRIFGRLGNYPA
LNWFFLGGLIGPVLVWLLQKAFPTKTWISQINLPVLLGATAAMPPATSVNFNCWIIVGVIFNYFVFKYCK
KWWQRYNYVLSAALDAGLAFMGVLLYFSLTMNGISINHWWGAKGENCPLASCPTAPGVLVDDFTVFFFFL
KIFVPFVNKNRLNDFLSMYLLY

FIG. 9

MDKIRAVISGGEKPPVDTDNDHNTDFEADRKMPDLDIVVSKSQEFDPVTSHLVNDIMEDEYAAVHVEDDS
PYPEVRAAVPSTDDPTLPQNTIRAWVIGLILTTVGCGMNMLFSFHSPSFAITTFVTSILAWPIGNFWAWI
VPDWKIFGASLNPGPFNVKEHTIITIMANVSFGTGAATATDILLAQNMFYKSNFGWGYNLLLIWSTQCIG
FAFGGVMRRFVVDSPGAIWPSNLVTATFLTNMHINENHTANGWKISRLAFFVIVFVASFVWYWFPGYIFQ
ALSYFSWITWIKPNNVIINQVFGSSSGLGMIPNNIALDWNQIAGYIGSPLIPPASVIATIFGSIVLIFWI
VVPAIHYSNTWYSQYLPISSTGSFDRFQQTYNVSKIIDHKTLSFNEAEYKKYSPLFLSTTFAISYGLSFA
SILATITHTICFHGRDLIASLKAKEKPDVHNRLMKAYKPVPEWWYLVVFLVFFGMSIATVRAWPTEMPVW
GLVFALIIAIIFLLPVAIIYAKTNIAVGLNVVTEFIVGYVLGGRPLCMMLFKTFGYITNNQAVTFVQDMK
LGHYMKIDPRTLFWAQFAATIWGSLVQIAVLEWAYGAIDNLCAADQKNHYTCPNGKVFFNASIIWGVIGP
QRQFSHGQIYYGLLFFFIIGAVTPVINWLILKKWPNSPVKYLHWPVFFSGTGYIPPATPYNYTSYCAVGL
FFGWWIKKKWFHWWSKYNYSLSAGLDIGLAWCSLIIFLCLSLTNTDFPSWWGNDVINTTLDTQVVTNIRH
ILKEGEAFGPSSW

FIG. 10

MSTIYRESDSLESEPSPTPTTIPIQINMEEEKKDAFVKNIDEDVNNLTATTDEEDRDPESQKFDRHSIQE
EGLVWKGDPTYLPNSPYPEVRSAVSIEDDPTIRLNHWRTWFLTTVFVVVFAGVNQFFSLRYPSLEINFLV
AQVVCYPIGRILALLPDWKCSKVPFFDLNPGPFTKKEHAVVTIAVALTSSTAYAMYILNAQGSFYNMKLN
VGYQFLLVWTSQMIGYGAAGLTRRWVVNPASSIWPQTLISVSLFDSLHSRKVEKTVANGWTMPRYRFFLI
VLIGSFIWYWVPGFLFTGLSYFNVILWGSKTRHNFIANTIFGTQSGLGALPITFDYTQVSQAMSGSVFAT
PFYVSANTYASVLIFFVIVLPCLYFTNTWYAKYMPVISGSTYDNTQNKYNVTKILNEDYSINLEKYKEYS
PVFVPFSYLLSYALNFAAVIAVFVHCILYHGKDIVAKFKDRKNGGTDIHMRIYSKNYKDCPDWWYLLLQI
VMIGLGFVAVCCFDTKFPAWAFVIAILISLVNFIPQGILEAMTNQHVGLNIITELICGYMLPLRPMANLL
FKLYGFIVMRQGLNLSRDLKLAMYMKVSPRLIFAVQIYATIISGMVNVGVQEWMMHNIDGLCTTDQPNGF
TCANGRTVFNASIIWSLPKYLFSSGRIYNPLMWFFLIGLLFPLAVYAVQWKFPKFKFAKHIHTPVFFTGP
GNIPPSTPYNYSLFFAMSFCLNLIRKRWRAWFNKYNFVVMGAGVEAGVAISVVIIFLCVQYPGGKLSWWGN
NVWKRTYDNDYKKFYTLKKGETFGYDKWW

FIG. 11

MSETVKDKVIIDEKVSTKGTVDYAEGAEYSERLSNHSSDFSQWYTDEQILHFMKKLGYENRTLYDIPEDV
AYILKKMPELTLEDSFKILKDSIIYFKDDENIPHDQYEEWKRLVDLEDLDSKEGIDEYDSFDIRAFASAI
KFHSPYQEVRAVVDPEDDPTIPVETFRAYFLAIIWSVIGSGFNEFFSHRVVSISLNTPIIQMFLYICGKA
WAKTIPCWTITIRGRKYGINIDKPWTQKEQMFSTLLYAICQGAFYTHYNILTQKLFYHSAFSFGYQFLLS
LSVQFIGFGFAGILRKFVVYPARALWPTVMPTIAINKALLGKEKHESGMSRYKFFFLTFFIMFIYNWFPT
YIINILNTFNWMTWIKPSNINLANITGGVTGLGINPISSFDWNVISFNSPLVYPFWSYLTQYLGCILAAL
IVIAVYYSNYMSCQYLPIFTNSLYTNTGHSFKVTEVLDSDNKLDVKKYQSYSPPYYSAGNLVSYGAFICA
YPLMITWSFIVHSKLLFNAFKDWALNLWAMRKLKSWVTMFKSDYRALDDYDDPHSNAMKNYKEVPDWWYF
AILIGSLVVGIAVVEHYPTNTPVWGLFVCLGFNFVFLIPTTILQATTGYSFGLNLLIEMVMGYALPGNPI
AIMILKAFGYNIDGQADNYVSNLKIAHYCKIPPMALFRGQCVIVFIQIFVNLGVLNWQISNIKDFCTPHQ
NAKFTCPDAVTYYNASVVWGAIGPKRIFNYIYPIFKWCWLIGACIGIFFGVWKRWGKFYPRYFDPMLFVG
GMLNMSPPYNLMYYTSGMIVSYISQYYMKRHHLNLWEKYNYVLSAGFSTGLVLSAIIIFFAVQYKDTAFN
WWGNTVPYAGADGVGYPLKNITDTANGYFGYAPGHYP

FIG. 12

MTARNSASIPTSIRKTSENEVSGDETPAGVGNLSTKTASKTSLTFRQSSSDESTSSYSGNHHNINIQHHP
NRPFRTNSSSFSPNDYSISESPSKSKKDGVHVSAVQLDNETDSEVESEVEELERELEAIEDSVYPEVRAA
VNPTDDVNLPVNTWRTWVLTTIFVIVFAAVNQFFSLRYPALSISFIVAQLILFPLGKLLNLLPNWKIGYG
RFSFYLNSSPFNVKEHAAITIAVSLTSSTAYATNILSAQTSFYKQNLSWGYKILLIVLTSQMLGYGFAGLT
RRWIVYPAAMIWPQTLVSTVLFRTLHGNSGNDIGVLKNNRISANGWTISRYRFFAYVMIGSFVFYWFPGF
IFKGLSYFTVLCWIWPKNRVVNQLFGYNSGLGILPLTFDWQQVVYNSNPLASPWWVICNTFGSVVLIFWI
VVPILYYKGVWFSNYLPMLSSSTFDHTGVSYNSSRVLNSDYSFNHTKYESYSPLYMPMSYSMSTALNFAA
VTAIFTHCALYNGKDIWQRLWKESGKDECIHRKLMRNYKEAPQWWYATLFIVVFGLTIFTVRYYDTQCPV
WALIVALLIFIVNFIPQGVLEGITNQHVGLNIITELIGGYILPGKPLANLMIKLYGFIPMRQGLEFSRDL
KLAQYMKIPPRILFFVQLFATILGGITQVAVQEWMNYHIPGICTTSQSNGFTCPNGRSIYNASLIWGAIG
PAKMFSKGKPYYPLIFFFLIGAVAPFITWGLRKRFPKSWIGKLNAPVLFTGPGNIPPATGINYSSWAIVG
FIFNYVIRKRAIHWWRKYNYVVLAAAMDSGVAVAGVVIFLCVSYPGGKITWWGNTVYTKTYDWKSVPYRSL
GPNETFGYTNW

FIG. 13

MKTPKFITYVTRGFKGLESKSVENNKDHIVENSSPIASKFHEFDEQKKSFEIINYAGHEKFVDDITERES
SVPGNAVYDITVRDIDAIVPVTDDVDIPASTFRMWILAFGLATVIAGVDAFFLMRYPSVSIAAIVALLVA
YPLGQLWYYIIPQWEIKLPRGIRVSLNPGRFNRKEHACLYIFVNICVSAKLVNTLIIEQIKFFGVNIGIG
RAILFNLCSYLSSFGWSGLALPILVYPPTLIWPSVLSSCALFKIFHDNDNTKACNWTISRLRYFFIVFVA
SFIWYWFPDLIFPALSSLGAWISWCKPSSAVLSQIFGVKTGLGLFPLTLDWAQISSLSNPLITPWWATCC
IFTSFVFWIWIVLPGLYYQNYWQVAHFPIMTNSIYTVSGKSYDAQKVVDSKWELVTQKYQEYSPVMLPIA
FIINIALSLGAFSSMMISFFLRFPTDVIQPICHVFKYSDIHTKLLKKYKRVHWGFYLASIIVSLGLGFAF
TEGWHDIQIRSYGFVVSMVIGAALYIPLSLIESRSSFTISMQAFFEIVAAFWFNGQPMALLYFYSFGFGT
LQHAMHMTQSAKIGHYMKVPPRLVAALLFTSGIWSSLVNSAVTGWIMYHVRDVCTSNAENNMTCRSPKTQ
FNSHLIWGLVGNHIFSSDGRYSFVMWFFLVGAVVSVVVYLLQISFPKSSWKHVNPALLLGGAAQIPSVTG
INYSTWAAVAFCFNYLIRRGYYSWWKKYNLITAAAMDCGVAIAGLFIYFCVVYTGGSSNFSWWGTTVSSA
GCDKKGCAHLSVSDISKPSGW

FIG. 14

MIGSINESPIEEHMNDSPSTKEKADSVDISDYIVSHSDDSLSKDIKKDTKSFLDVEHGEISTVDEFEEDS
PYPEVRAAVPPTDDPSMPCNTIRMWTIGLIYSTVGAAVNMFFSLRNPTVTLSVLISELLAYPALQIWDLI
FPDREFRIGRLKFNFKPGPFNVKEHALIVVMSSVSFGNAYSTDIILAQRVHYKQRFGFGYEICLTLATQL
IGYGLAGLSRRLLVRPASMLWPVNLVQCTLIKTLHRKDLRNAVANGWRISPFRFFLYVFIASFIWNWFPS
YIFQALSLFAWVTWIRPNSPTVNQIFGESTGISILPMTFDWNQISAYILSPLMAPADALMNILLGVILFF
WIVTPALNFTNTWYGDYLPISSSGIIDHFGNSYNVTRILTKDATFDLDAYQNYSPIFMSTTYALAFGLSF
ASITSVIFHVILYHGKEIYDRLRDPPAPDIHEKLMKAYDEVPFYWYLSVFLAFFGMMMGTIYGWKTETPW
WVIIVGVIFSAVWFIPIGIVQAITNIQLGLNVFTEFIVGYMYPGRPLAMMIFKTVGYITMTQGLAFAADL
KEGHYMKLPPRIMFYTQMIATIWSCFVQIGVLDWALGNIDNVCQADQPDNYTCPNATVFFNSSVIWGVIG
PKRMFSGKNTYTGLQYFWLAGVLGTILFWALWKKWPQKWWGQLNGPLIFGGTGYIPPATPVNYLAWSGIG
LFFNYYLKKIFADWWQKYNFTLSALDTGTQLSVIILFFCLQLPMVNFPDWWGNDGAFNTLDATGAAVRKL
VNESAR

FIG. 15

```
atgagtacca tttataggga gagcgactcg ttggagtcgg agccctcgcc   50
aacgccaaca accattccta tccagatcaa tatggaagag gaaaagaaag  100
atgctttcgt taagaatatt gacgaggacg tcaataatct cactgcgact  150
actgatgagg aggaccgcga tccggaaagc caaaaattcg acaggcattc  200
catacaggag gaaggtctcg tttggaaggg cgaccctaca tacttgccca  250
attctccata tcctgaagtg agatcggcgg tgtccatcga ggatgacccc  300
accatccgcc tcaaccactg gagaacgtgg ttcttgacca cggtgtttgt  350
ggtagttttc gccggtgtta atcaattttt ttccctgaga tatccatcgc  400
tagagatcaa cttccttgtt gcacaagttg tttgctaccc aattggtagg  450
atactggctc tcttgcccga ctggaagtgt tctaaagtgc catttttcga  500
tttaaacccg ggcccattta ccaaaaagga acacgccgtg gtcacaattg  550
ccgtggcgct tacttcctct actgcatacg ctatgtacat tttgaacgcc  600
cagggaagct tttacaacat gaaacttaat gtcggatatc agttcttgtt  650
ggtttggaca tctcaaatga ttggttatgg tgctgcaggt cttaccagaa  700
gatgggtcgt caaccctgca agctctatct ggcctcagac tttaatttca  750
gtgtccttgt ttgattcgtt gcactcgaga aaagttgaaa agacagtcgc  800
aaatggttgg acgatgcccc gttacaggtt cttcttaatc gtccttatcg  850
gatcgttcat ctggtattgg gtacctggat tcctctttac cggtctgtcc  900
tatttcaacg ttatcctatg ggggtcgaag acaagacaca atttcatcgc  950
taacacaatc tttggtactc aaagtggtct cggtgcgttg ccaattacat 1000
ttgactacac ccaggtttcc caagccatgt ccggctctgt tttcgccaca 1050
ccattctacg tctccgccaa cacctatgca tcagtgttga tattcttcgt 1100
catagtgctg ccatgtcttt attttacgaa tacctggtat gccaaataca 1150
tgcccgtcat ttcaggttct acttatgaca acactcaaaa caaatacaac 1200
gtaacaaaga ttcttaacga ggattattcc attaatcttg agaaatataa 1250
ggaatactca ccggtattcg ttccattttc ctatcttttg tcgtatgctt 1300
taaattttgc cgctgttatc gccgtttttg tccactgcat cttataccac 1350
ggtaaagata ttgtcgccaa gtttaaagac cgtaaaaatg gtggcactga 1400
cattcacatg agaatctact ccaagaacta taaggattgt cccgattggt 1450
ggtatttact tttgcagatt gtcatgatcg gtttaggatt tgtagcagtg 1500
tgctgtttcg atactaagtt cccagcttgg gcatttgtta ttgcaatatt 1550
aatttccctt gtaaatttca tcccgcaagg tatcttggaa gcaatgacta 1600
```

FIG. 17A

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accaacacgt aggtttgaat attatcacag aattgatctg cggttatatg 1650
ctgcctttaa gaccaatggc aaacttatta ttcaagctat acggatttat 1700
tgtcatgaga caaggcttga atttgagtag agatttgaaa ttagccatgt 1750
acatgaaggt ttcccctcgt ttgatctttg ccgttcaaat ctatgccact 1800
atcatatcag gcatggttaa cgttggtgtc caggaatgga tgatgcataa 1850
tatcgatggc ttatgtacca ccgatcaacc aaatggcttc acttgtgcta 1900
atggtcgcac ggttttcaat gcttccatta tctggtcttt gccaaagtat 1950
cttttctcat cagggcgcat ttataatccg ctgatgtggt tcttcttgat 2000
tggtttgcta ttcccactag ccgtttatgc tgttcaatgg aaattcccta 2050
aatttaaatt tgctaagcac attcatactc ctgtattttt cacaggccca 2100
ggtaatattc caccaagcac accttataac tactcattat tttttgcaat 2150
gtcattctgc ctaaacttga taagaaaaag atggagagct tggttcaata 2200
agtacaattt cgtcatgggg gccggtgttg aagcaggtgt ggcaatctcc 2250
gtcgtcatca tcttcttgtg tgtacagtac ccaggtggta agctcagctg 2300
gtggggaaac aacgtttgga aaagaacgta tgataatgat tataaaaaat 2350
tttatacctt aaagaaaggt gagacatttg gttatgataa atggtggtaa 2400
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Nucleic acid sequence of S. cerevisiae OPT1

FIG. 17B

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  50 ATGAGTGAAA CAGTCAAAGA TAAAGTTATA ATTGATGAGA AGGTATCCAC
  51 AAAAGGTACT GTTGATTACG CCGAGGGTGC TGAGTATTCT GAGAGGCTTT
 101 CAAATCATTC ATCAGACTTT TCTCAGTGGT ATACGGATGA ACAGATACTG
 151 CACTTTATGA AGAAGCTGGG TTATGAAAAT CGCACTCTTT ATGATATTCC
 201 GGAAGACGTT GCGTATATCC TCAAAAAAAT GCCTGAATTG ACACTTGAGG
 251 ATTCCTTCAA AATACTAAAA GACTCTATCA TCTATTTCAA GGATGATGAG
 301 AACATTCCAC ACGATCAATA TGAGGAGTGG AAGAGATTGG TTGACTTGGA
 351 GGACTTGGAT TCAAAAGAGG GGATAGATGA ATATGATAGC TTTGACATTA
 401 GAGCATTTGC TTCTGCTATT AAATTTCATT CGCCTTACCA AGAGGTTAGA
 451 GCTGTTGTTG ATCCAGAAGA TGATCCCACC ATTCCAGTGG AGACATTCCG
 501 AGCATATTTT CTGGCAATAA TTTGGTCTGT CATCGGTTCA GGATTTAATG
 551 AGTTTTTTTC ACACAGGGTG GTTTCAATTT CACTGAATAC TCCAATTATC
 601 CAAATGTTTT TATATATCTG TGGTAAGGCT TGGGCTAAAA CTATCCCCTG
 651 TTGGACTATA ACCATCAGGG GCAGAAAGTA TGGTATCAAT ATCGATAAAC
 701 CATGGACCCA AAAAGAGCAA ATGTTTTCAA CCTTGTTATA TGCAATTTGT
 751 CAAGGCGCGT TCTATACTCA TTACAATATT CTAACGCAAA AACTCTTTTA
 801 CCATTCTGCT TTCTCGTTTG GCTACCAATT TTTACTTTCG TTATCCGTAC
 851 AATTTATTGG ATTTGGATTT GCTGGCATCC TTAGAAAATT CGTTGTTTAT
 901 CCAGCCCGTG CACTATGGCC AACAGTCATG CCAACTATTG CTATCAACAA
 951 GGCACTATTG GGTAAAGAAA AGCATGAATC TGGAATGAGC AGGTATAAAT
1001 TCTTTTTCTT GACTTTTTTT ATCATGTTCA TCTATAACTG GTTTCCCACT
1051 TACATTATTA ATATTCTAAA CACTTTCAAT TGGATGACCT GGATCAAGCC
1101 AAGTAACATT AATCTCGCAA ACATCACGGG AGGAGTCACT GGTCTTGGGA
1151 TTAATCCTAT CTCATCTTTT GACTGGAATG TTATTTCGTT TAATTCTCCT
1201 TTAGTTTACC CATTTTGGTC ATACTTAACA CAATATCTTG GTTGCATATT
1251 AGCAGCTTTA ATTGTTATTG CAGTATACTA TAGTAATTAT ATGAGTTGCC
1301 AATACCTGCC AATATTCACA AATTCTTTGT ATACTAATAC TGGCCATTCC
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FIG. 18A

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1351 TTTAAAGTTA CTGAGGTATT AGACAGTGAC AATAAGCTAG ATGTGAAAAA
1401 ATATCAAAGC TACTCGCCAC CATACTATAG TGCTGGAAAT TTGGTATCAT
1451 ATGGTGCTTT CATTTGCGCA TATCCTCTGA TGATTACATG GTCGTTTATT
1501 GTACACTCAA AGTTATTGTT CAATGCTTTC AAAGATTGGG CTTTGAATTT
1551 GTGGGCCATG AGAAAACTTA AATCTTGGGT CACAATGTTC AAAAGCGATT
1601 ACAGGGCGCT CGACGATTAT GATGACCCAC ATTCTAATGC CATGAAAAAC
1651 TATAAAGAAG TTCCAGATTG GTGGTATTTT GCCATATTGA TAGGTTCACT
1701 TGTTGTTGGA ATAGCTGTTG TAGAGCACTA CCCAACAAAT ACACCAGTTT
1751 GGGGTCTTTT TGTTTGTTTA GGATTTAATT TTGTTTTCTT GATTCCAACT
1801 ACTATCCTTC AAGCAACCAC TGGTTATTCG TTTGGTTTGA ATCTACTAAT
1851 TGAAATGGTG ATGGGGTACG CTTTACCAGG TAATCCAATC GCCATAATGA
1901 TTTTGAAGGC TTTTGGTTAT AACATCGACG GCCAAGCAGA TAATTACGTT
1951 TCTAACTTAA AAATAGCGCA TTATTGTAAG ATTCCGCCAA TGGCGCTATT
2001 CAGGGGACAA TGTGTTATAG TTTTCATTCA GATATTTGTC AATCTAGGTG
2051 TTCTGAATTG GCAAATCTCC AATATCAAAG ACTTTTGCAC ACCTCATCAA
2101 AACGCAAAAT TCACCTGTCC TGATGCTGTG ACCTACTATA ATGCTTCCGT
2151 TGTCTGGGGT GCAATTGGGC CAAAAAGAAT TTTCAATTAC ATTTATCCAA
2201 TATTTAAATG GTGTTGGTTG ATAGGCGCAT GCATTGGCAT ATTTTTTGGT
2251 GTTTGGAAGC GCTGGGGTAA GTTTTATCCC AGATATTTTG ACCCAATGTT
2301 ATTTGTAGGT GGAATGCTTA ATATGAGCCC TCCATATAAC CTGATGTATT
2351 ACACTTCTGG TATGATTGTT AGTTACATTT CCCAGTACTA CATGAAAAGA
2401 CACCATTTAA ATCTGTGGGA GAAATATAAT TATGTTTTAT CGGCAGGCTT
2451 TTCAACGGGC TTGGTTTTAT CAGCTATTAT CATTTTCTTT GCTGTCCAAT
2501 ATAAAGACAC AGCTTTTAAT TGGTGGGGCA ATACAGTTCC GTATGCTGGT
2551 GCCGATGGCG TTGGCTATCC TCTAAAGAAC ATAACTGATA CAGCAAATGG
2601 CTATTTCGGC TATGCTCCAG GACACTATCC ATGA
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Nucleic acid sequence of YPR194c

FIG. 18B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#3

Art Unit	:	
Examiner	:	
Serial No.	: 09/914,541	
Filed	: 8/29/01	
Inventors	: Jeffrey Becker	
	: Melinda Hauser	
	: Amy Donhardt	Docket No.:1046-PCT-US-00
	: David Barnes	
Title	: EUKARYOTIC PEPTIDE	Confirmation No.: 7890
	: UPTAKE SYSTEM FOR	
	: TRANSPORTATION OF	
	: ENKEPHALINS	Dated: November 29, 2001

LETTER

Box PCT
Commissioner for Patents
P. O. Box 2327
Arlington, VA 22207

Sir:

We acknowledge receipt of the Notification of Missing Requirements dated October 22, 2001, copy enclosed.

We also submit herewith a computer readable copy and a printed copy of the sequence listing and thereby fulfills the requirements of 37 C.F.R. §§ 1.821-1.825. Also enclosed is a Statement Accompanying Sequence Listing.

Also enclosed is the executed Combined Declaration along with our check in the amount of $130.00 comprising the surcharge for filing the Combined Declaration later than the date of actual filing of the application in the U.S. Patent & Trademark Office.

Respectfully submitted,

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